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How can the cystic fibrosis respiratory microbiome influence our clinical decision making?

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Purpose of review

Almost 15 years have now passed since bacterial community profiling techniques were first used to analyse respiratory samples from people with cystic fibrosis. Since then, many different analytical approaches have been used to try to better understand the contribution of the CF lung microbiota to disease, with varying degrees of success. We examine the extent to which CF respiratory microbiome research has been successful in informing clinical decision making, and highlight areas that we believe have the potential to yield important insight.

Recent findings

Recent research on the CF lung microbiome can be broadly divided into efforts to better characterise microbiota composition, particularly relative to key clinical events, and attempts to understand the CF lung microbiology as an interactive microbial system. The latter, in particular, has led to the development of a number of models in which microbiome-mediated processes precipitate clinical events.

Summary

Growing technological sophistication is enabling increasingly detailed microbiological data to be generated from CF respiratory samples. However, translating these data into clinically useful measures that accurately predict outcomes and guide treatments remains a formidable challenge. The development of systems biology approaches that enable the integration of complex microbiome and host-derived data provide an exciting opportunity to address this goal.

Keywords: microbial ecology, pulmonary exacerbations, systems biology

Cystic fibrosis (CF) lung infections have been the focus of intense research and clinical interest since their first description by Dorothy Andersen and others in the 1940s [1]. However, since that time, this field has been a source of equal measures of information and confusion. To illustrate, the ecology of these infections, and therefore the "pathogens" that have been the focus of treatments, laboratory tests, and clinical trials, have changed over time, with an early focus on *S. aureus* transitioning to *P. aeruginosa* and others. This ecology continues to change [2]. Amidst this shifting landscape, clinical microbiology has tried to establish tests that will faithfully diagnose the cause of clinical change, such as the isolation of a specific bacterial species, or will reliably identify effective antibiotic treatments. While epidemiologic relationships have been identified between specific bacteria and clinical decline on a population scale, these efforts have not been as successful at the level of the individual patient. By the early 2000s, as the field surveyed this confusing state of affairs, many researchers began to investigate new methods to detect and characterize the microbiology of the CF airway.

The emergence of human microbiome research, a field that aims to understand the role of complex microbial systems in health and disease, raised the hope of rapid advances in the clinical management for individuals with cystic fibrosis (CF). However, despite this field still being in its relative infancy, there has been frustration that microbiome-based studies are yet to yield substantial improvements in respiratory care. In part, this frustration is the natural consequence of the hubris that accompanies technological breakthroughs. However, we would argue that microbiome research in CF has, in some respects, lost its way.

First, it is important to be clear about what we mean by *microbiome research*. Microbiome research has developed in parallel to advances in culture-independent microbiology. As a result, the term *culture-independent*, which refers to a characteristic of the technologies used, and *microbiome research*, which refers to a conceptual framework, have at times been mistakenly treated as interchangeable. In their simplest form, culture-independent techniques

can be seen as an extension of the culture-based approaches used to detect pathogens in respiratory samples. In contrast, microbiome-centric approaches treat a polymicrobial airway infection not as a collection of separate populations, but as a microbial system with its own distinct characteristics.

Culture-independent assays for specific pathogens, particularly those based on simple PCR-based tests, have proven highly successful and have been integrated into routine diagnostics of various types. The ability of these approaches to directly detect and quantify specific organisms within clinical samples is especially useful where diagnostic information is needed rapidly and for pathogens that are difficult to culture *in vitro*. For example, PCR-based assays are commonly employed to detect respiratory viruses and, to a lesser extent, fungal pathogens and fastidious or slow-growing bacteria. However, CF microbiology more generally has continued to rely almost exclusively on culture, in part due to its ready ability to report bacterial phenotypes thought to be clinically important (such as mucoidy for *P. aeruginosa*), but also because it has been considered to be important to isolate representative pathogens for antibiotic susceptibility testing.

In the context of CF, there is ample evidence from *in vitro* susceptibility testing alone that the standard approach to choosing antibiotics is ineffective. Perhaps the most common method involves isolating individual colonies of the "traditional pathogens" that grow on laboratory media (including *P. aeruginosa*, *S. aureus*, and a few other species), reculturing these isolates in another, rich medium under aerobic conditions, and testing susceptibilities to added antibiotics. A number of studies have shown that this approach, and even more complex versions involving multiple antibiotics and biofilm susceptibilities, do not reliably identify treatments that lead to clinical improvement among patients with the best-studied traditional pathogen, *P. aeruginosa*.

These and other clinical outcomes studies underscore the many inadequacies of culture-based diagnostic microbiology. Other weaknesses of the inadequacy of conventional clinical laboratory culture are well-recognised, not least of which is a limited ability to grow *in vitro* certain groups of commonly abundant microbes, such as anaerobic bacteria [3-5]. This shortcoming sparked interest in whether techniques used to characterise complex environmental microbial systems might be able to report a wider a range of organisms in samples from the CF airways. Instead of targeting pathogen-specific genomic sequences, these approaches typically amplify regions of the 16S rRNA gene that are common to all bacteria. The identities and relative abundances of the bacteria present can then be inferred from the intervening variable sequences [6].

16S rRNA gene terminal restriction fragment length polymorphism (T-RFLP) analysis, an approach developed originally to characterise polymicrobial communities in environmental systems such as soil, bioreactor sludge, and termite guts [7,8], was found to be readily applicable to CF sputum [9-11]. Importantly, these early investigations revealed far greater bacterial diversity in the CF lung than suggested by culture-based analysis, including the widespread abundance of genera that are refractory to growth under standard diagnostic conditions for CF samples, including *Veillonella*, and *Prevotella* [10,12].

Following these early studies, the sophistication of the profiling techniques available grew rapidly. Large-scale clone sequencing [13,14] and the use of PhyloChips [15] supported the findings of the early primarily electrophoretic approaches, while the emergence in 2008 of the first next-generation sequencing platforms allowed 16S rRNA gene amplicons to be characterised in far greater depth [16]. All of these approaches have been consistent in reporting groups of microbes that were not generally associated previously with CF respiratory disease, particularly those that are able to exploit lower oxygen tensions, including the genera

Prevotella, *Veillonella*, *Streptococcus*, and *Gemella*. In addition, analysis of lung tissue obtained surgically demonstrated that these organisms did not simply represent sample contamination during passage through the heavily colonised upper airways [17].

Despite detecting a far broader range of bacteria than diagnostic culture, microbiota profiling approaches failed to make the transition from the research laboratory to diagnostic service. The time required to perform the analysis and the need to convert microbiota data into a readily interpretable format represented significant hurdles. However, the most substantial obstacle was arguably a lack of clarity around how microbiota information could be factored into clinical decision-making. Despite the fact that the evidence for any direct contributions to lung disease for many of traditional CF pathogens is, at best, conflicting and limited, species that have been reported through culture-independent approaches have generally been considered clinically irrelevant. Moreover, the culture approaches used by CF clinical laboratories are designed to select against many of the species identified as abundant in sputum by microbiota approaches under the assumption that they were either contaminants or otherwise unimportant, despite some being considered pathogens in other contexts. In light of the increasing number of species commonly reported in CF airway samples, a more objective basis for designating detected microbes as targets for treatment- whether the goal is to favour or inhibit those taxa- is clearly needed.

Tracking shifts in airway microbiology

While not yet contributing directly to clinical decision-making, the increased application of microbiome analytical approaches continues to develop our understanding of CF airway microbiology. In particular, these studies have demonstrated a phenomenon that is well-recognised within environmental microbiology but largely absent from clinical thinking: that *the physicochemical characteristics of a site determine the composition of the microbial*

communities that they contain. This concept - that the tissue environment selects its microbiota - contrasts with the common clinical axiom that infection risk is explained largely by exposure, and holds that the characteristics of the airway microbiota will change with the progression of lung disease and the types of therapy employed. Indeed, the application of microbiota-characterisation approaches has allowed the changes in airway microbiology that occur with disease progression to be detailed in a relatively unbiased, more inclusive fashion. For example, it is now known that the CF lung microbiota increases in diversity with the establishment of chronic infection during early-life [18,19]. This likely reflects the build-up of respiratory secretions, which provide both nutritional resources and a growth environment within the lung. During childhood and adolescence, increasing antibiotic exposure and airway inflammation is associated with a fall in lung microbiota diversity, and the growing abundance of species regarded as pathogens, such as *H. influenzae, S. aureus,* and *P. aeruginosa* [20-28]. Finally, there is typically an almost complete collapse in community diversity during end-stage lung disease, with the lung microbiome often becoming completely dominated by specific bacteria, including *Achromobacter*, *Burkholderia*, or *P. aeruginosa* [29,30]. In this schema, the relationship between disease progression and airway microbiota can be multidirectional: Increasingly obstructed airways and cumulative antibiotic exposure select for, and create niches for, characteristic microbiota, and interactions between community members, and between the community and the host, can in turn drive both disease and change in microbiota constituency.

This ability to see the impact of lung disease or treatment strategies reflected in airway microbiota composition allows us to better understand historical changes in typical CF airway microbiology. For example, recent changes in CF clinical care are likely to have contributed, directly and indirectly, to the increasing prevalence of *Burkholderia multivorans*, *Stenotrophomonas maltophilia*, *Alcaligenes* (*Achromobacter*) *xylosoxidans*, methicillin-

resistant *Staphylococcus aureus*, *P. aeruginosa* (including multidrug-resistant *P. aeruginosa*), and *Mycobacterium abscessus* complex in the CF population [31-35]. Recent studies have described the impact of specific therapies on the CF lung microbiome, such as inhaled aztreonam [36], and such approaches are likely to prove particularly valuable in understanding the impact of the widespread deployment of CFTR modifiers and potentiators. This new generation of therapies could fundamentally alter the characteristics of airway disease for many individuals with CF, and while their influence on lung microbiology is still unclear, a recent study of ivacaftor treatment has reported a rapid and substantial reduction in sputum bacterial abundance, with a particularly marked effect on *P. aeruginosa* levels [37].

An ecological perspective

Although microbiota profiling techniques have provided a useful way to characterise the changing airway microbiology in CF, it is important to recognise that they were not developed simply to extend the coverage provided by culture-based approaches. Rather, their use stemmed from interest in a concept from environmental microbiology: that *the behaviour of complex microbial systems cannot be understood by examining their constituent species in isolation*. This concept reflects nature. Single species are seldom present in any natural environment. Instead, microbes almost invariably exist in complex interacting communities in which synergistic and competitive metabolic relationships, interspecies communication, the production of antimicrobial compounds, and the manipulation of the shared growth environment, all contribute to microbiome characteristics [38]. A microbiome-centric approach is well-accepted when characterising systems such as the human gut [38], but though these approaches have been under-utilised in CF lung infections, they have clear potential to provide greater insight into the fundamental mechanisms that drive CF lung disease.

The potential of interspecies interactions to influence clinical outcomes by shaping the behaviour of key pathogens is well-documented. Duan and colleagues demonstrated using a rat infection model that members of the oropharyngeal microbiota could substantially enhance the lung damage caused by *P. aeruginosa* [39]. This work was later extended to show the proportion of oropharyngeal bacterial taxa that could significantly increase the pathogenicity of *P. aeruginosa*, despite being avirulent or beneficial when present in isolation [40]. Interactions between members of the CF lung microbiota also help to define the characteristics of the microbiome as an integrated system. Two important characteristics are *resistance*, the degree to which microbial composition remains unchanged in response to a disruptive force, such as antibiotic exposure, and *resilience*, the rate at which microbial composition returns to its original composition after being disrupted [41]. Both resistance and resilience are strongly influenced by the number, nature, and strength of inter-species relationships. Cuthbertson and colleagues recently illustrated this by showing that the composition of the CF respiratory microbiota as a whole is highly resistant during the onset and treatment of exacerbations, while both core and rare taxa show substantial resilience following cessation of therapy [42]. These complex qualities of the unified microbiota would not have been evident by studying the dynamics of individual, isolated taxa such as *P. aeruginosa*, which in this case would simply have shown temporary decreases during antibiotic treatment [43].

Efforts to identify the mechanisms by which the airway microbiome influences lung disease have been particularly focused on pulmonary exacerbations, which, despite their clinical importance, remain poorly understood. Analyses of the airway microbiota during the transition from relative clinical stability to acute exacerbation, and vice versa, have failed to report consistent changes in bacterial community composition [21,26,27,42,44-46]. Many recent investigations have therefore focused on the behaviour of the airway microbiota, rather than its compositional characteristics, particularly with regard to the influence of *P. aeruginosa*. This

process has led to the development of a range of conceptual mechanistic models of how the respiratory microbiome might precipitate pulmonary exacerbations. For example, one model proposes that exacerbations stem from the occasional blooming of cells from pseudomonal biofilms [47,48]. While triggering an acute immune response, these episodically biofilm-dispersed cells are likely to have a greater capacity to kill macrophages and escape phagocytosis [49] but be more responsive to antibiotic therapy [47,48]. Such bloom events could also lead to the expansion of infection to new areas of the lung, contributing to a failure to recover baseline lung function [26]. The fact that these blooms might constitute only a very small proportion of total bacterial numbers might explain why bacterial or pseudomonal levels appear not to increase during exacerbation onset [45]. Factors strongly influenced by the activities of the wider airway microbiota, such as nutrient availability, oxygen tension, and phage activity, can trigger pseudomonal biofilm dispersal [50], suggesting that in such a model, pseudomonal bloom might be mediated by the wider respiratory microbiome. Other models are also associated with *P. aeruginosa*. One such model involves the precipitation of exacerbations by increases in pseudomonal production of the toxic phenazine compound, pyocyanin. Pyocyanin not only acts as an alternative electron acceptor, facilitating pseudomonal growth under low oxygen conditions within mucus plugs [51], but it can also damage the airways directly [52]. Venkataraman and colleagues demonstrated that *in vitro* common fermentation products from co-habitant anaerobic bacteria, particularly 2,3-butanediol, significantly increased pyocyanin production, exotoxin release, and biofilm formation by *P. aeruginosa* [51]. Analysis of breath gas metabolites from a small number of individuals suggested that levels of 2,3-butanedione are higher in CF patients compared to their healthy counterparts, and that this compound may reach levels that can damage the airways directly [53]. Longitudinal analysis in a single patient showed levels of 2,3-butanedione fell during intravenous antibiotic treatment for exacerbations [53]. As broad-spectrum antibiotics

disproportionately impact non-pseudomonal members of the airway microbiota [54], the resulting reduction in their production of 2,3-butanedione or related exoproducts might therefore be a substantial contributor to clinical benefit associated with therapy. Whether shifts in metabolic relationships between the airway microbiota and *P. aeruginosa* can precipitate exacerbation onset however remains unclear.

In vitro and *in vivo* observations have hinted at other potential mechanistic pathways. For example, utilisation of peptidoglycan shed by gram-positive bacteria enhances the virulence of *P. aeruginosa* in co-culture [55]. Investigations using animal models suggest that peptidoglycan sensing allows *P. aeruginosa* to express traits that modify microbial community composition, as well as enhancing pathogenicity [55]. Another interesting observation is that *Gemella*, a genus of facultative anaerobes generally found in low abundance in sputum, appears to increase in abundance at exacerbation relative to baseline [56]. Based on the keystone-pathogen hypothesis [57], certain low-abundance microbial pathogens can orchestrate inflammatory disease by remodelling a normally benign microbiota into a dysbiotic one, suggesting that the ability of *Gemella* to fulfil such a role warrants further investigation. Yet another proposal involves the so-called climax–attack model [58]. In this model, two major functional communities coexist in the CF lung: an attack community that consists of transient viral and other microbial populations that induce strong innate immune response, and a climax community that is slower-growing and inherently resistant to antibiotic therapy [59]. However, this hypothesis awaits more supportive evidence from clinical specimens.

Interest in inter-kingdom interaction as a mediator of pathogenesis in the CF lung has also grown steadily. Interactions between bacterial and fungal pathogens in the CF airways are already well-known. For example, the phenazines secreted by *P. aeruginosa* are known to inhibit the growth of *Aspergillus fumigatus* at high concentrations but promote growth at sub-inhibitory concentrations [60], while pseudomonas-derived volatile organic compounds can

trigger invasion of the lung parenchyma by *A. fumigatus* [61]. The recent description of complex fungal communities that showed evidence of adaptation to the CF airways and stability during pulmonary exacerbation or treatment [62], suggests such interactions maybe be widespread.

The increasing complexity of data that can now be generated with a range of different 'omics technologies has led to interest in using systems biology approaches to integrate microbiome-derived and immunological data [63]. For example, such an approach has been employed recently to determine the relationship between nasopharyngeal microbiota characteristics and host immune response in children experiencing infection by respiratory syncytial virus [64]. However, even with the development of such integrative analytical strategies, testing models of microbiome-mediated pathogenesis in CF remains challenging. The characteristics of lung disease, pulmonary exacerbations, and airway microbiology differ substantially within and between patients. Increasing the size of study cohorts, given such interpatient variability, is therefore likely to provide only limited additional insight, unless an appropriate system of patient subgrouping can be devised. The difficulty of reliably obtaining representative samples from the lower airway is also a continuing frustration, with samples such as sputum and bronchoalveolar lavage losing the original spatial distribution of the microbes that they contain, a potentially important contributor to microbiome function [65]. Even where representative airway samples are available, identifying and distinguishing between the effects of numerous separate synergistic or antagonistic pathways that may operate concurrently is extremely difficult, a problem compounded by the ability of different bacteria to perform the same activities by the same or different pathways.

Therapeutic opportunities

The use of microbiome data to guide clinical decision-making in CF remains a distant goal. However, research being performed in other microbiome contexts has highlighted strategies by which the microbial communities can be manipulated to influence the behaviour of key pathogens. For example, many different bacterial species produce the quorum sensing signal molecule autoinducer-2 (AI-2), allowing it to act as a basis for inter-species communication [66]. Manipulation of AI-2 production by individual bacterial community members can alter microbiome structure and function [67], while the enzymatic quenching of AI-2-mediated interspecies communication can block pathways associated with pathogenic phenotypes such as virulence and biofilm formation [66,68-71]. Other strategies by which microbiome-mediated pathogenesis might be controlled, such as the degradation of metabolic mediators, are also worthy of investigation.

Importantly, were approaches such as those described to prove clinically effective, they might provide an opportunity to reduce the burden of antibiotics in CF care. While we know that antibiotics are both safe and effective in the short-term, in the longer-term they are likely to contribute to the prevalence of bacteria with a high natural tolerance, such as *P. aeruginosa.* The combined use of conventional antimicrobial treatments with strategies that disrupt microbiome-mediated pathogenesis might therefore provide both short- and long-term benefits.

Conclusions

The application of a new generation of technologies to the analysis of CF lung infections, together with a suite of ecological concepts that were refined for environmental microbiology but are relatively novel within clinical microbiology, has revealed hugely complex microbial communities. After a decade of CF microbiota research, it is natural to be disappointed that these findings have not yet found their way into clinical care. However, this disappointment should be weighed against the scale of the challenges that we face within this still emerging

field. The assessment made by Dr. Efraim Racker in 1985, prior to the advent of microbiome analysis in CF, that “Anyone who reviews the literature on CF and isn't confused, is confused.” [72] perhaps now applies doubly in the era of systems biology. However, the opportunity to gain a fundamental understanding of the mechanisms driving CF lung disease, coupled with the rapidly advancing technical capacities of the microbiome field, will continue to drive efforts to define and model microbiome-mediated pathogenesis.

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